Procedural and Strain-related Variables Significantly Affect Outcome in a Murine Model of Focal Cerebral Ischemia

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THE RECENT AVAILABILITY of transgenic mice has led to a burgeoning number of reports describing the effects of specific gene products on the pathophysiology of stroke. Although focal cerebral ischemia models in rats have been well described, descriptions of a murine model of middle cerebral artery occlusion are scant and sources of potential experimental variability remain undefined. We hypothesized that slight technical modifications would produce widely discrepant results in a murine model of stroke and that controlling surgical and procedural conditions could lead to reproducible physiological and anatomic stroke outcomes. To test this hypothesis, we established a murine model that would permit either permanent or transient focal cerebral ischemia by intraluminal occlusion of the middle cerebral artery. This study provides a detailed description of the surgical technique and reveals important differences among strains commonly used in the production of transgenic mice. In addition to strain-related differences, infarct volume, neurological outcome, and cerebral blood flow appear to be importantly affected by temperature during the ischemic and postischemic periods, mouse size, and the size of the suture that obstructs the vascular lumen. When these variables were kept constant, there was remarkable uniformity of stroke outcome. These data emphasize the protective effects of hypothermia in stroke and might help to standardize techniques among different laboratories to provide a cohesive framework for evaluating the results of future studies in transgenic animals. (Neurosurgery 38:523–532, 1996)

Key words: Cerebral ischemia, Focal, Hypothermia, Intraluminal, Mouse, Strain

The recent advent of genetically altered mice provides a unique opportunity to evaluate the role of single gene products in the pathophysiology of stroke. Although there is an increasing number of reports about the effect of cerebral ischemia in transgenic mice, there are no detailed descriptions of the murine models involved nor is there a detailed analysis of potentially important procedural variables that may affect stroke outcome. Most descriptions of a murine model (1, 4, 8, 9, 14, 17–19, 23, 25) are devolved descriptions of the widely used rat models of focal cerebral ischemia (22, 26). Although there has been some attention paid to strain-related differences in the susceptibility of mice to cerebral ischemia (4), few technical considerations have been addressed in published studies. Because our pilot data demonstrated that minor differences in operative procedure or postoperative care translated into major differences in stroke outcome, the current study was undertaken to systematically identify important surgical, technical, and anatomic considerations required to obtain consistent results in a murine model of focal cerebral ischemia. When strokes are created in a rigidly controlled manner, differences caused by the absence (or overexpression) of a single gene product should be readily discernable.

We present a detailed rendering of a reproducible murine model of focal cerebral infarction based on modifications of the original rat model (26). We identify those procedural variables that have a large impact on stroke outcome and that have not been previously reported in technical descriptions of murine stroke models. These variables include suture length and gauge, methods of vascular control, temperature regulation in mice, and differences among strains commonly used in the breeding of transgenic animals. Because the model we describe lends itself to the study of either permanent or transient focal cerebral ischemia, we present evidence that with carefully chosen ischemia times, infarct volume and mortality in reperfused animals can be made to approximate those seen

with permanent occlusion. Understanding potential model-dependent sources of variability in stroke outcome can help to clarify divergent results among different laboratories. The adoption of a standardized model that yields consistent results is an important first step toward the use of transgenic mice in the study of the pathophysiology of stroke.

MATERIALS AND METHODS

Animal purchase and anesthesia

Male mice of three different strains (C57 BlackJ6, CD-1, and 129J) were purchased from Jackson Laboratories (Bar Harbor, ME). The animals were 8 to 10 weeks old and weighed between 18 and 37 g (as indicated) at the time of the experiments. Each mouse was anesthetized with an intraperitoneal injection of 0.3 ml of ketamine (10 mg/ml) and xylazine (0.5 mg/ml). An additional dose of 0.1 ml was administered before the withdrawal of the occluding suture in animals undergoing transient ischemia. On the day after surgery, anesthesia was repeated immediately before laser Doppler flow measurements were obtained and the animals were killed. These procedures have been approved by the Institutional Animal Care and Use Committee at Columbia University and are in accordance with the American Association for Laboratory Animal Science guidelines for the humane care and use of laboratory animals.

Surgical set-up

Each animal was positioned supine on a gauze pad that rests on a temperature-controlled operating surface. A rectal temperature probe (Yellow Springs Instruments, Inc., Yellow Springs, OH) was inserted to regulate the temperature of the operating surface and to maintain a constant animal core temperature of 36 to 38°C. To facilitate exposure, the right hindpaw and left forepaw were taped to the operating surface, the right forepaw was taped to the animal's chest, and the tail was taped to the rectal probe (*Fig. 1A*). A midline neck incision was made by gently lifting the loose skin between the manubrium and the jaw and excising a 1-cm^2 circle of skin. The paired midline submandibular glands directly underlying this area were bluntly divided, with the left gland left in situ. The right gland was retracted cranially with a small straight Sugita aneurysm clip (Mizutto America, Inc., Beverly, MA) and secured to the table by a 4-0 silk ligature and tape. The sternocleidomastoid muscle was then identified, and a 4-0 silk ligature was placed around the belly. This ligature was drawn inferolaterally and taped to the table to expose the omohyoid muscle covering the carotid sheath. The exposure is shown in *Figure 1B*.

Operative approach

Once the carotid sheath was exposed, the mouse and the temperature-controlled surface were placed under an operating microscope (16–25× zoom; Zeiss, Thornwood, NY); a coaxial light source was used to illuminate the field. Under magnification, the omohyoid muscle was carefully divided with pickups. The common carotid artery (CCA) was carefully freed from its sheath, taking care not to apply tension to the vagus nerve (which runs lateral to the CCA). Once freed, the CCA was isolated with a 4-0 silk ligature and taped loosely to the operating table. Once proximal control of the CCA was obtained, the carotid bifurcation was placed in view. The occipital artery, which arises from the proximal external carotid artery (ECA) and courses posterolaterally across the proximal internal carotid artery (ICA) to enter the digastric muscle, was isolated at its origin, coagulated using a Malis bipolar microcoagulator (Codman-Schurtleff, Randolph, MA), and divided with microscissors. This enabled better visualization of the ICA as it coursed posteriorly and cephalad underneath the stylohyoid muscle toward the cranial base. Just before the ICA enters the cranium, it gives off a pterygopalatine branch, which courses laterally and cranially. This branch was identified, isolated, and divided at its origin, during which time the CCA-ICA axis straightened. A 4-0 silk suture was then placed around the ICA for distal control, the end of which was loosely taped to the operating surface.

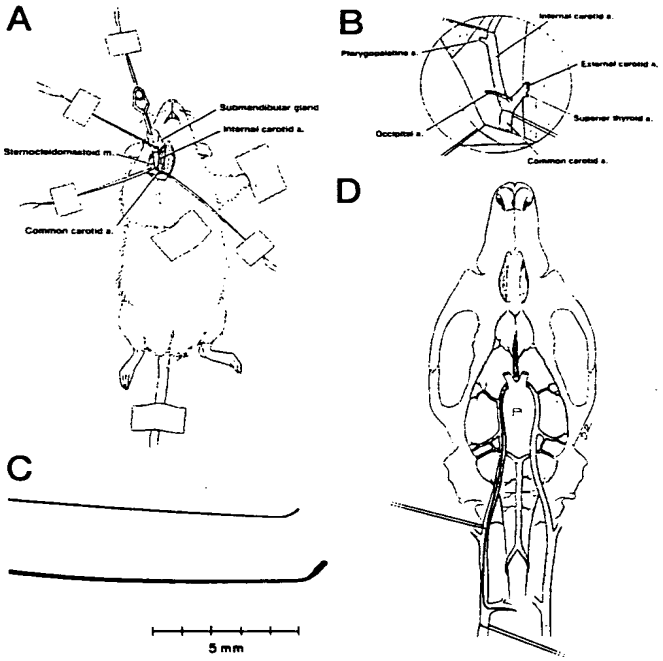

FIGURE 1. Overview of operative set-up for murine focal cerebral ischemia model. *A*, suture-based retraction system is shown in the diagram (*a.*, artery; *m.*, muscle). *B*, view through the operating microscope. The large vascular stump represents the ECA, which is situated inferomedially in the operating field (*a.*, artery). *C*, photograph of the heat-blunted occluding suture of the indicated gauge (5-0 [*bottom*] or 6-0 [*top*] nylon). *D*, schematic diagram of murine cerebrovascular anatomy, with occluding suture in the anterior cerebral artery, occluding the MCA at its point of origin.

The ECA was then placed in view. Its craniomedial course was skeletonized, and its first branch, the superior thyroid artery, was cauterized and divided. Skeletonization was subsequently performed distally by elevation of the hyoid bone to expose the artery's bifurcation into the lingual and maxillary arteries. Just proximal to this bifurcation, the ECA was cauterized and divided. Sufficient tension was then applied to the silk sutures surrounding the proximal common and distal internal carotid arteries to occlude blood flow, with care taken not to traumatize the arterial wall. Tape on the occluding sutures was readjusted to maintain occlusion.

Introduction and threading of the occluding intraluminal suture

Immediately after carotid occlusion, an arteriotomy was fashioned in the distal external carotid wall, just proximal to the cauterized area. Through this arteriotomy, a heat-blunted 5-0 or 6-0 nylon suture (as indicated under Results) was introduced (*Fig. 1, C* and *D*). As the suture was advanced to the level of the carotid bifurcation, the ECA stump was gently retracted caudally, directing the tip of the suture into the proximal ICA. Once the occluding suture entered the ICA, tension on the proximal and distal control sutures was relaxed and the occluding suture was slowly advanced up the ICA toward the cranial base under direct visualization (beyond the level of the cranial base, sight of the occluding suture tip is lost). Localization of the distal tip of the occluding suture across the origin of the middle cerebral artery (MCA) (proximal to the origin of the anterior cerebral artery) was determined by the length of suture chosen (12 or 13 mm, as indicated under Results) (*Fig. 1C*), by laser Doppler flowmetry (see Ancillary Physiological Procedures), and by staining of the cerebral vasculature after the animals were killed (see below). After the placement of the occluding suture was complete, the ECA stump was cauterized to prevent bleeding through the arteriotomy once arterial flow was reestablished.

Completion of surgical procedure

For all of the experiments shown, the duration of carotid occlusion was less than 2 minutes. To close the incision, the sutures surrounding the proximal and distal CCA, as well as the sternocleidomastoid muscle, were cut and withdrawn. The aneurysm clip was removed from the submandibular gland, and the gland was laid over the operative field. The skin edges were then approximated with one surgical staple, and the animal was removed from the table.

Removal of the occluding suture to establish transient cerebral ischemia

The transient cerebral ischemia experiments required reexploration of the wound to remove the occluding suture. For each of these experiments, initial wound closure was performed with a temporary aneurysm clip rather than a surgical staple, to provide quick access to the carotid. Proximal control with a 4-0 silk suture was reestablished before removal of the occluding suture, to minimize bleeding from the ECA stump. During removal of the occluding suture, cautery of the ECA stump was begun early, before the distal suture had completely cleared the stump. Once the suture was completely removed, the stump was more extensively cauterized. Reestablishment of flow in the extracranial ICA was confirmed visually, and the wound was closed as for permanent focal ischemia described above. Confirmation of intracranial reperfusion was accomplished with laser Doppler flowmetry (see Ancillary Physiological Procedures).

Calculation of infarct volume

Twenty-four hours after MCA occlusion, the surviving mice were subsequently anesthetized with 0.3 ml of ketamine (10 mg/ml) and xylazine (0.5 mg/ml). After final weight, temperature, and cerebral blood flow (CBF) readings were recorded (as described below), the animals were perfused with 5 ml of a 0.15% solution of methylene blue and saline to enhance visualization of the cerebral arteries. The animals were then decapitated, and the brains were removed. Brains were inspected for evidence of correct catheter placement, as evidenced by negative staining of the vascular territory subtended by the MCA, and placed in a mouse brain matrix (Activational Systems Inc., Warren, MI) for 1-mm sectioning. The sections were immersed in 2% 2,3,5-triphenyltetrazolium chloride in 0.9% phosphate-buffered saline, incubated for 30 minutes at 37°C, and placed in 10% formalin (5). After 2,3,5-triphenyltetrazolium chloride staining, infarcted brain was visualized as an area of unstained (white) tissue in a surrounding background of viable (brick red) tissue. Serial sections were photographed and projected on tracing paper at a uniform magnification; all serial sections were traced and cut out, and the paper was weighed by a technician blinded to the experimental conditions. Under these conditions, infarct volumes were proportional to the summed weights of the papers circumscribing the infarcted region and were expressed as a percentage of the right hemispheric volume. These methods have been validated in previous studies (2, 12, 15, 16).

Ancillary physiological studies

Ancillary physiological studies were performed on each of the three different strains used in the current experiments, immediately before and after the operative procedure. Systemic blood pressures were obtained by catheterization of the infrarenal abdominal aorta and were measured using a Grass Model 7 polygraph (Grass Instrument Co., Quincy, MA). An arterial blood sample was obtained from this infrarenal aortic catheter; arterial pH, pCO_2 (mm Hg), pO_2 (mm Hg), and hemoglobin oxygen saturation (%) were measured using a Blood Gas Analyser and Hemoglobinometer (Grass Instrument Co.). Because of the need for arterial puncture and abdominal manipulation to measure these physiological parameters, animals were designated solely for these measurements (stroke volumes, neurological outcome, and CBF were not measured in these same animals).

Transcranial measurements of CBF were made using laser Doppler flowmetry (Perimed, Inc., Piscataway, NJ), after reflection of the skin overlying the calvarium, as previously described (10) (transcranial readings were consistently the

same as those made after craniectomy in pilot studies). To accomplish these measurements, the animals were placed in a stereotactic head frame, after which they underwent midline skin incision from the nasion to the superior nuchal line. The skin was swept laterally, and a 0.7-mm straight laser Doppler probe (Model PF2B) was lowered onto the cortical surface, wetted with a small amount of physiological saline. Readings were obtained 2 mm posterior to the bregma, both 3 and 6 mm to each side of midline, using a sterotactic micromanipulator and keeping the angle of the probe perpendicular to the cortical surface. Relative CBF measurements were made immediately after anesthesia, after occlusion of the MCA, and immediately before the animals were killed and are expressed as the ratio of the Doppler signal intensity of the ischemic compared with the nonischemic hemisphere. For animals subjected to transient cerebral ischemia, additional measurements were made just before and just after withdrawal of the suture, initiating reperfusion.

The surgical procedure/intraluminal MCA occlusion was considered to be technically adequate if ≥50% reduction in relative CBF was observed immediately after the placement of the intraluminal occluding catheter (15 of the 142 animals used in this study [10.6%] were excluded because of inadequate drop in blood flow at the time of the occlusion). These exclusion criteria were shown in preliminary studies to yield levels of ischemia sufficient to render consistent infarct volumes by 2,3,5-triphenyltetrazolium chloride staining. Reperfusion was considered to be technically adequate if CBF at catheter withdrawal was at least twice occlusion CBF (13 of 17 animals in this study [76%]).

Temperature

Core temperature during the peri-infarct period was carefully controlled throughout the experimental period. Before surgery, a baseline rectal temperature was recorded (Yellow Springs Instruments Model 74 Thermistemp rectal probe, Yellow Springs Instruments). Intraoperatively, temperature was controlled using a thermocouple-controlled operating surface. After MCA occlusion, the animals were placed in an incubator for 90 minutes, with animal temperature maintained at 37°C, using the rectal probe connected via thermocouple to a heating source in the incubator. Temperature was similarly controlled in those animals subjected to transient ischemia, including a 45-minute (ischemic) period as well as a 90-minute postischemic period in the incubator. After placement in the core-temperature incubator, the animals were returned to their cages for the remaining duration of observation before the animals were killed.

Neurological examination

Before the administration of anesthesia at the time the animals were killed, the mice were examined for obvious neurological deficits, using a four-tiered grading system as follows: 1) normal spontaneous movements; 2) animal circling toward the right; 3) animal spinning to the right; 4) animal crouched on all fours, unresponsive to noxious stimuli. This system was shown, in preliminary studies, to accurately predict infarct size and is based on systems developed for use in rats (6).

Data analysis

Stroke volumes, neurological outcome scores, CBF, and arterial blood gas data were compared using an unpaired Student's t test. Values are expressed as means ± standard error of the mean, with $P < 0.05$ considered statistically significant. Mortality data, when presented, were evaluated using χ^2 analysis.

RESULTS

Effects of strain

Three commonly used mouse strains (CD1, C57/Bl6, and 129J) were used to compare the variability in stroke outcome after permanent focal cerebral ischemia. To establish that there were no gross anatomic differences in collateralization of the cerebral circulation, the circle of Willis was visualized using India ink in all three strains (*Fig. 2*). These studies failed to reveal any gross anatomic differences. Mice of similar sizes (20 ± 0.8 g, 23 ± 0.4 g, and 23 ± 0.5 g for 129J, CD1, and C57Bl mice, respectively) were then subjected to permanent focal ischemia under normothermic conditions using a 12-mm length of 6-0 nylon occluding suture. Significant strain-related differences in infarct volume were noted, with infarcts in 129J mice being significantly smaller than those observed in CD1 and C57/Bl6 mice despite identical experimental conditions (*Fig. 3A*). Differences in infarct size were paralleled by neurological examination, with the highest scores (i.e., the most severe neurological damage) being seen in the C57/Bl6 and CD1 mice (*Fig. 3B*).

To determine the relationship between infarct volume and CBF to the core region, laser Doppler flowmetry was performed through the thin murine calvarium. No preoperative

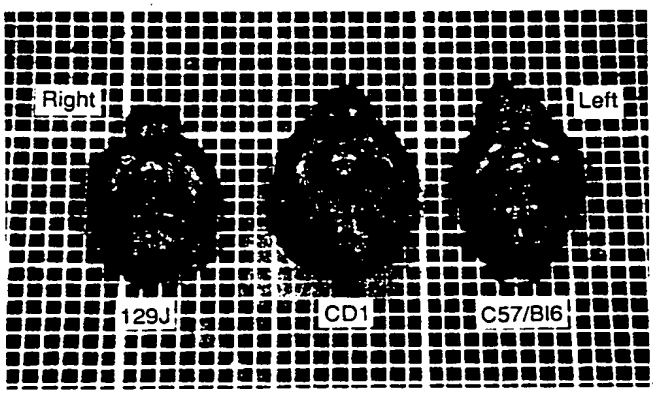

FIGURE 2. Comparison of cerebrovascular anatomy between strains of mice. After receiving anesthesia, the mice were given an intracardiac injection of India ink and were then killed. An intact circle of Willis can be observed in all strains, including bilateral posterior communicating arteries, indicating that there are no gross strain-related differences in cerebrovascular anatomy.

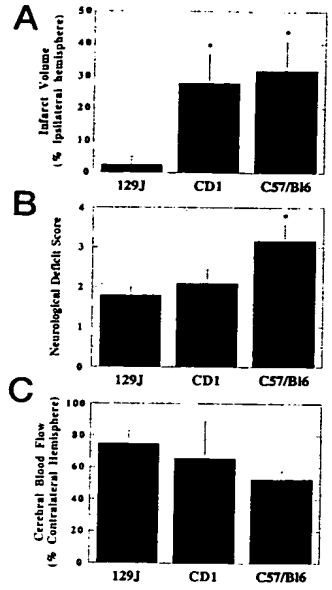

FIGURE 3. Effects of mouse strain on stroke outcome. Mice (males weighing 20–23 g) were subjected to permanent MCA cclusion (using 12-mm 6-0 occluding suture), and indices of stroke outcome were determined. *A*, effects of strain on infarct volume, determined as a percentage of ipsilateral hemispheric volume, as described under Materials and Methods. *B*, effects of strain on neurological deficit score, graded from no neurological deficit (Grade 0) to severe neurological deficit (Grade 4), with scores determined as described under Materials and Methods. *C*, effects of strain on CBF, measured by laser Doppler flowmetry as relative flow over the infarcted territory compared with blood flow over the contralateral (noninfarcted) cortex. Strains included 129J (n = 9), CD1 (n = 11), and C57/Bl6 mice (n = 11); *, $P < 0.05$ versus 129J mice.

strain-related differences in CBF were observed, corresponding to the lack of gross anatomic differences in vascular anatomy (*Fig. 2*). Measurement of CBF immediately after insertion of the occluding catheter revealed that similar degrees of flow reduction were created by the procedure (the percentage of ipsilateral/contralateral flow immediately after insertion of the obstructing catheter was 23 ± 2%, 19 ± 2%, and 17 ± 3% for 129J, CD1, and C57/Bl6 mice, respectively). Not surprisingly, blood flow to the core region measured at 24 hours before the animals were killed was lowest in those animals with the most severe neurological injury (*Fig. 3C*).

Anatomic and physiological characteristics of mice

Baseline arterial blood pressures, as well as arterial blood pressures after MCA occlusion, were nearly identical for all animals studied and were not effected by mouse strain or size (*Table 1*). Analysis of arterial blood for pH, pCO_2, and hemoglobin oxygen saturation (%) similarly revealed no significant differences (*Table 1*).

Effect of animal size and bore of the occluding suture

To investigate the effects of mouse size on stroke outcome, mice of two different sizes (23 ± 0.4 g and 31 ± 0.7 g) were subjected to permanent focal cerebral ischemia. To eliminate other potential sources of variability in these experiments, experiments were performed under normothermic conditions in mice of the same strain (CD1), using occluding sutures of

TABLE 1. Pre- and Postoperative Physiological Parameters[a]

Parameter	Preoperative	Sham	Stroke
MAP	102 ± 5.5	94 ± 1.9	88 ± 4.9
pH	7.27 ± 0.02	7.23 ± 0.04	7.28 ± 0.01
pCO_2	46 ± 1.3	44 ± 1.3	47 ± 3.5
O_2 Sat	89 ± 1.6	91 ± 1.8	85 ± 2.2
Hb	14.6 ± 0.42	14.3 ± 0.12	14.2 ± 0.12

[a] Preoperative, anesthetized animals before carotid dissection; Sham, anesthetized animals undergoing the surgery described in the text, immediately before introduction of the occluding suture; Stroke, anesthetized animals undergoing the surgery described in the text, immediately after introduction of the occluding suture; MAP, mean arterial pressure (mm Hg); pCO_2, partial pressure of arterial CO_2 (mm Hg); O_2 Sat, O_2 saturation (%); Hb, hemoglobin concentration (g/dl). P = not significant for all comparisons (data shown is for small, 22-g C57/Bl6 mice).

identical length and bore (12-mm 6-0 nylon). Under these conditions, small mice (23 ± 0.4 g) sustained consistently large infarct volumes (28 ± 9% of ipsilateral hemisphere). Under identical experimental conditions, large mice (31 ± 0.7 g) demonstrated much smaller infarcts (3.2 ± 3%, $P = 0.02$) (*Fig. 4A*), less morbidity at neurological examination (*Fig. 4B*), and a tendency to maintain higher ipsilateral CBF after infarction than smaller animals (*Fig. 4C*).

Because we hypothesized that the reduction in infarct size in these large animals was related to a mismatch in diameter/length between the occluding suture and the cerebral blood vessels, we fashioned longer/thicker occluding sutures (13-mm, 5-0 nylon) for use in these larger mice. Large CD1 mice (34 ± 0.8 g) that underwent permanent occlusion with these larger occluding sutures sustained a marked increase in infarct volumes (50 ± 10% of ipsilateral hemisphere, $P < 0.0001$, compared with large mice infarcted with the smaller occluding suture) (*Fig. 4A*). These larger mice infarcted with larger occluding sutures demonstrated higher neurological deficit scores (*Fig. 4B*) and lower ipsilateral CBF (*Fig. 4C*) compared with similarly large mice infarcted with smaller occluding sutures.

Effects of temperature

To establish the role of perioperative hypothermia on the infarct volumes and neurological outcomes after MCA occlusion, small C57/Bl6 mice (22 ± 0.4 g) were subjected to permanent MCA occlusion with 12-mm 6-0 gauge suture, with normothermia maintained for two different durations; Group 1 animals (normothermia) were operated on as described above, maintaining temperature at 37°C from the preoperative period until 90 minutes after occlusion. Group 2 animals (hypothermia) were maintained at 37°C from preoperation to only 10 minutes after occlusion, as has been described previously (14). Within 45 minutes after removal from the thermocouple-controlled warming incubator, the core temperature in this second group of animals dropped to 33.1 ± 0.4°C (and dropped further to 31.3 ± 0.2°C at 90 min). Animals operated on under conditions of prolonged normo-

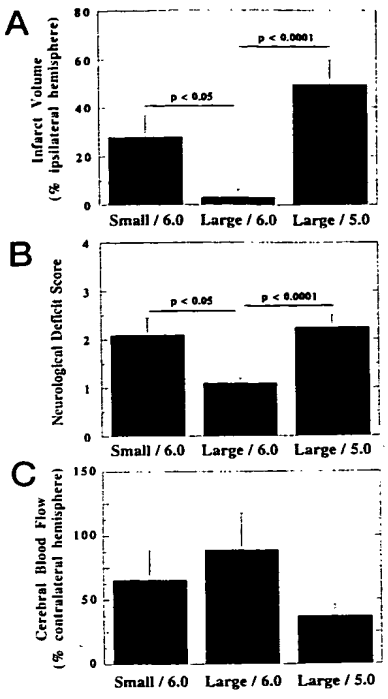

FIGURE 4. Effects of animal size and diameter of the occluding suture on stroke outcome. Male CD-1 mice of the indicated sizes were subjected to MCA occlusion permanent as described under Materials and Methods. Suture size (gauge) is indicated in each panel. Small animals (n = 11) were those between 20 and 25 g (mean, 23 g), and large animals were between 28 and 35 g (mean, 32 g) (n = 14 for 6-0 suture, n = 9 for 5-0 suture). *A*, effects of animal/suture size on infarct volume. *B*, neurological deficit score. *C*, CBF, measured as described in the legend to *Figure 3*. *P* values are as shown.

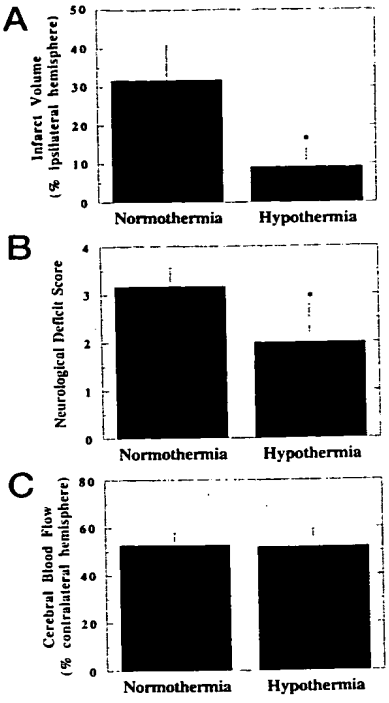

FIGURE 5. Effects of temperature on stroke outcome. Male C57/Bl6 mice were subjected to permanent MCA occlusion (6-0 suture) and then reperfusion. Core temperatures were maintained for 90 minutes at 37°C (normothermia, n = 11) using an intrarectal probe with a thermocouple-controlled heating device. In the second group (hypothermia, n = 12), the animals were placed in cages and were left at room temperature after an initial 10 minutes of normothermia (mean core temperature 31°C at 90 minutes). In both groups, after this 90-minute observation period, the animals were returned to their cages with ambient temperature maintained at 37°C for the duration of observation. Twenty-four hours after MCA occlusion, indices of stroke outcome were recorded. *A*, infarct volume. *B*, neurological deficit score. *C*, CBF, measured as described in the legend to *Figure 3*. *, $P < 0.05$.

thermia (Group 1) exhibited larger infarct volumes (32 ± 9%) than hypothermic animals (Group 2) (9.2 ± 5%, $P = 0.03$) (*Fig. 5A*). Differences in infarct volume were mirrored by differences in neurological deficit (3.2 ± 0.4 versus 2.0 ± 0.8, $P = 0.02$) (*Fig. 5B*) but were largely independent of CBF (52 ± 5 versus 52 ± 7, P = not significant) (*Fig. 5C*).

Effects of transient MCA occlusion

Because reperfusion injury has been implicated as an important cause of neuronal damage after cerebrovascular occlusion (24), a subset of animals was subjected to a transient (45 min) period of ischemia and then reperfusion as described above, and comparisons were made with those animals that underwent permanent MCA occlusion. The time of occlusion was chosen on the basis of preliminary studies (not shown), which demonstrated unacceptably high mortality rates (>85%) with 180 minutes of ischemia and rare infarction (<15%) with 15 minutes of ischemia. To minimize the confounding influence of other variables, other experimental conditions were kept constant (small [22.5 ± 0.3 g] C57/Bl6 mice were used, the occluding suture consisted of 12-mm 6-0 nyon, and experiments were performed under normothermic conditions). The initial decline in CBF immediately after occlusion was similar in both groups (16 ± 2% versus 17 ± 3%, for transient versus permanent occlusion groups, respectively, P = not significant). Reperfusion was confirmed by laser Doppler (2.3-fold increase in blood flow after the removal of the occluding suture to 66 ± 13%) and visually by intracardiac methylene blue dye injection in representative animals. Infarct sizes (29 ± 10% versus 32 ± 9%), neurological deficit scores (2.5 ± 0.5 versus 3.2 ± 0.4), and CBF measurements at the time the animals were killed (46 ± 18% versus 53 ± 5%) were similar between animals subjected to transient cerebral ischemia and reperfusion and those subjected to permanent focal cerebral ischemia (P = not significant for all groups) (*Fig. 6*).

DISCUSSION

The growing availability of genetically altered mice has led to an increasing use of murine models of focal cerebral ischemia to impute specific gene products in the pathogenesis of stroke. Although recent publications describe the use of an intraluminal suture to occlude the MCA to create permanent and/or transient cerebral ischemia in mice, there has been only scant description of the necessary modifications of the original technical report in rats (8, 13, 14, 17–19, 25, 26). The experiments described herein not only provide a detailed technical explanation of a murine model suitable for either

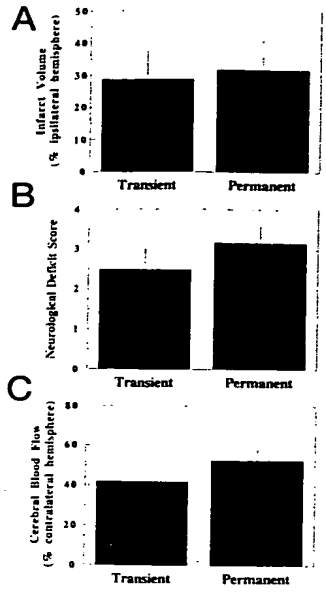

FIGURE 6. Outcome comparisons between permanent focal cerebral ischemia and transient focal cerebral ischemia and then reperfusion. The MCA was occluded either permanently (n = 11) or transiently (45 min, n = 17) with 6-0 suture in 22-g male C57/Bl6 mice, as described under Materials and Methods. Twenty-four hours after MCA occlusion, indices of stroke outcome were recorded. *A*, infarct volume. *B*, neurological deficit score. *C*, CBF, measured as described in the legend to *Figure 3*.

permanent or transient focal MCA ischemia but also address potential sources of variability in the model.

Importance of strain

One of the most important potential sources of variability in the murine cerebral ischemia model we describe is related to the strain of animal used. Our data suggest that of the three strains tested, 129J mice are particularly resistant to neurological injury after MCA occlusion. Although Barone et al. (4) similarly found differences in stroke volumes among three strains of mice (BDF, CFW, and BALB/C), these differences were ascribed to variations in the posterior communicating arteries in those strains. As anatomic differences in cerebrovascular anatomy were not grossly apparent in our study (*Fig.* 2), our data suggest that nonanatomic strain-related differences are also important in outcome after MCA occlusion.

Because stroke outcome differs significantly between two strains of mice (129J and C57/Bl6) commonly used to produce transgenic mice via homologous recombination in embryonic stem cells (11), our data suggest an important caveat to experiments performed with transgenic mice. Because early founder progenies from the creation of transgenic animals with these strains have a mixed 129J/C57/Bl6 background, experiments should be performed either with sibling controls or after a sufficient number of backcrossings to ensure strain purity.

Importance of size

We report here that larger animals require a longer and thicker intraluminal suture to sustain infarction volumes that are consistent with those obtained in smaller animals with smaller occluding sutures. Size matching of animal and suture appears to be important to produce consistent cerebral infarction, with too small a suture leading to insufficient ischemia and too large a suture leading to frequent intracerebral hemorrhage and vascular trauma (unpublished observation).

The use of animals of similar size is important not only to minimize potential age-related variability in neuronal susceptibility to ischemic insult but also to ensure that small differences in animal size do not obfuscate meaningful data comparison. In our studies, we demonstrate that size differences of as little as 9 g can have a major impact on infarct volume and neurological outcome after cerebral ischemia. Further experiments using a larger bore occluding suture in larger animals suggest that the increased propensity of smaller animals to have larger strokes was not because of a relative resistance of larger animals to ischemic neuronal damage but was because of the small size of the suture used to occlude the MCA in large animals. Although these data were obtained using CD1 mice, we have performed similar studies and found these results to be true for other mouse strains as well, such as C57/Bl6 (unpublished data). Previously published reports use mice of many different sizes (from 21 to 35 g), as well as different suture diameters and lengths, which are often unreported (14, 17). Our studies indicate that animal and suture size are important methodological issues that must be addressed in scientific reports.

Importance of temperature

It has long been recognized that hypothermia protects a number of organs from ischemic injury, including the brain. Studies performed in rats have demonstrated that intraischemic hypothermia up to 1 hour after MCA occlusion is protective (3, 15), reducing both mortality and infarct volumes with temperatures of 34.5°C. Although these results have been extrapolated to murine models of cerebral ischemia in that studies often describe maintenance of normothermia in animals, the post-MCA occlusion temperature-monitoring periods have been extremely brief ("immediately after surgery" or "10 minutes after surgery") (4, 14). Our results indicate that animals fail to autoregulate their temperature beyond these brief durations, becoming severely hypothermic during the postoperative period, and that temperature differences up to 90 minutes after MCA occlusion can have a profound effect on indices of stroke outcome after MCA occlusion (longer durations of normothermia were not studied). Although others have ensured normothermia, using a feedback system based on rectal temperature similar to the one we describe, the duration of normothermia is often not specified (17). Our results argue for clear identification of methods for monitoring and maintaining temperature, as well as the durations involved, so that experimental results can be compared both within and between centers studying the pathophysiology of stroke.

Transient versus permanent occlusion

The pathophysiology of certain aspects of permanent cerebral ischemia may well be different from that of cerebral ischemia and then reperfusion; it was therefore important that a model be described that permitted analysis of either condition. Although differences between these two models were

not extensively tested in the current series of experiments, under the conditions tested (45 min of ischemia and then 23 h of reperfusion), no significant differences were found in any index of stroke outcome. Variable durations of ischemia and reperfusion have been reported in other murine models of transient cerebral ischemia, with ischemic times ranging from 10 minutes to 3 hours and reperfusion times ranging from 3 to 24 hours (17, 25). Studies in rats have shown that short periods of ischemia and then reperfusion are associated with smaller infarcts than permanent occlusion (20, 21, 24). However, as the duration of ischemia increases beyond a critical threshold (between 120 and 180 min), reperfusion is associated with larger infarcts (7, 21, 26). For the current series of experiments, the durations of ischemia and reperfusion were chosen so as to obtain infarcts comparable to those observed after permanent MCA occlusion, which is likely to explain why we failed to show differences between permanent and transient ischemia. These durations in the transient model were chosen after pilot experiments revealed that shorter ischemic durations (15 min) rarely led to infarction, whereas 180 minutes of occlusion and then reperfusion led to massive infarction and nearly 100% mortality within 4 to 6 hours in normothermic animals (unpublished observation). Although indices of stroke outcome may be measured earlier than 24 hours, we elected the 24-hour observation time because observation at this time permits the study of delayed penumbral death, which is likely to be clinically relevant to the pathophysiology of stroke in humans. Furthermore, a 24-hour observation time has been shown in a rat model to be sufficient for full infarct maturation (2, 12, 15, 16).

Technical aspects of the murine model

Technical aspects of the surgery needed to create focal cerebral ischemia in mice differ in certain important respects from that in rats. Self-retaining retractors, which have been advocated for use in rats in previous reports (26), are unwieldy in mice. Suture-based retraction secured with tape provides a superior alternative. In rats, clip occlusion of the proximal and distal carotid artery after mobilization of the ECA has been reported (26) but creates more carotid trauma and hemmorhage in mice. Without distal ICA control, which has not been previously described in mice, backbleeding from the ECA is consistently uncontrollable. Using the techniques described in this article, surgery can be completed with virtually no blood loss, which is especially important given the small blood volume in mice.

Unlike the rat model, the occlusion and transection of the ECA branches and the pterygopalatine artery in the murine model is achieved with electrocautery and microscissors. Previous reports of murine surgery have been unclear as to whether the pterygopalatine artery was taken (17, 25). Others have described a method with permanent occlusion of the CCA and transcarotid insertion of the suture without attention to either the external carotid system or the pterygopalatine artery. Although effective for permanent occlusion, this latter method makes reperfusion studies impossible.

The method of reperfusion originally described in the rat requires blind catheter withdrawal without anesthesia (26). When we attempted this in pilot studies in mice, several animals hemorrhaged. We therefore have developed a method of suture removal under direct visualization in the anesthetized animal, which not only allows us to visually confirm reperfusion of the extracranial carotid artery but affords meticulous hemostasis. Furthermore, we were able to perform immediate pre- and post-reperfusion laser Doppler readings in the anesthetized animal.

These laser Doppler readings are similar to those described by Kamii et al. (17) and Yang et al. (25) in that they are made intermittantly and with the use of a stereotactic micromanipulator. They differ, however, in that the coordinates we use (2 mm posterior and 3 and 6 mm lateral to the bregma) are slightly more lateral and posterior than the previously published core and penumbral coordinates (1 mm posterior and 2 mm and 4.5 mm lateral to the bregma). These coordinates, which we adopted based on pilot studies, are the same as those used by Huang et al. (14).

CONCLUSION

These studies demonstrate specific technical aspects of a murine model of focal cerebral ischemia and reperfusion, which should enable considerable reproducibility of measurements between different laboratories. In addition, these studies provide a framework for understanding important procedural variables that can greatly impact stroke outcome, which should lead to a clear understanding of nonprocedure-related differences under investigation. Most importantly, this study emphasizes the need for careful control of mouse strain, animal and suture size, and temperature in experimental and control animals. Conditions can be established so that stroke outcome is similar between models of permanent focal cerebral ischemia and transient focal cerebral ischemia, which should facilitate direct comparison and permit the study of reperfusion injury. The model described in this study should provide a cohesive framework for evaluating the results of future studies in transgenic animals and to facilitate an understanding of the role of specific gene products in the pathophysiology of stroke.

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COMMENTS

Prompted by the development of transgenic mice and the possible effects of specific gene products on the pathophysiology of stroke, Connolly et al. have performed a carefully controlled study, which highlights certain precautions that must be taken by those using a murine model of stroke. When using the intravascular suture occlusion technique, size of the mouse, size of the suture, strain of the mouse, and temperature are important variables that must be controlled. The authors describe the technical aspects in sufficient detail to make them readily reproducible by others. This is a useful contribution to stroke research.

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It is unfortunate that most studies of experimental stroke are not preceded by such careful analyses of the animal model such as those presented by Connolly et al. Inherent variability in small and large animal stroke models has produced inconclusive and conflicting results for a variety of therapies. In a murine model of reversible middle cerebral artery occlusion, these authors showed the critical importance of several variables in determining infarct volume, including animal size, temperature, and size of the occluding intraluminal suture. The detailed description of the experimental protocol should enable other investigators to use this technique.

Murine models for stroke and other cerebrovascular disorders are increasingly important as transgenic mice are developed. Although the protocol described in this study produced consistent infarct volumes, it should be noted that nearly 40% of animals were discarded because of inadequate middle cerebral artery occlusion or lack of reperfusion. An additional unreported percentage of animals died or were eliminated because of technical failures (e.g., intracerebral hemorrhage). This does not diminish the importance of this model; researchers need to breed colonies large enough to accommodate these occurrences.

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ANNOUNCEMENT

Structured Abstracts Requirement for *Neurosurgery* Submissions

As of January 1, 1996 all manuscripts submitted for consideration to *Neurosurgery* are required to include an abstract consisting of no more than 250 words structured into "specific" sections. Each section must be comprised of a heading and a statement. For the sake of brevity, descriptions under each section heading need not be in complete sentences and may be formulated in phrases.

Abstracts for *General Clinical* and *Experimental* papers should include the following sections: **Objective, Methods, Results,** and **Conclusion**. Abstracts for papers that are significantly technical in nature should include a **Technique/Technical Development** and/or **Instrumentation** section(s), as appropriate. Abstracts for *Case Reports* follow the above format but should contain **Objective and Importance, Clinical Presentation, Intervention** (or **Technique**), and **Conclusion** headings. Abstracts will be critically reviewed and evaluated for direct and accurate conciseness to the manuscript's content.

Note the following as examples and clarifications:

CLINICAL/EXPERIMENTAL PAPERS
Objective—State the primary objective of and rationale for the study; include the importance of the issue being addressed.
Methods—Define the basic design, procedures, and/or setting in which the study was conducted.
(**Instrumentation**—Describe the instrument(s) being presented/investigaged.)
(**Technique** or **Technical Development**—Describe the significance of the technique being presented and/or the significant technical aspects of the paper.)
Results—Present significant data and observations gathered.
Concluson—Interpret findings and give principle conclusions; recommend clinical approach and/or need for future investigations.

CASE REPORTS
Objective and Importance—State significance of issue and importance of the case(s).
Clinical Presentation—Describe case(s) presented, pertinent attendant issues, and observations.
Intervention—Describe course of treatment.
(**Technique**—Describe technique or therapeutic approach.)
Conclusion—State outcome and recommended guidance pathways.

The *Information for Contributors* details instructions for developing structured abstracts for all general contributions.

For other useful information regarding this topic, please refer to the following articles:

1. Ad Hoc Working Group for Critical Appraisal of the Medical Literature: A proposal for more informative abstracts of clinical articles. **Ann Intern Med** 106:598–604, 1987.
2. Haynes RB, Mulrow CD, Huth EJ, Altman DG, Garner MJ: More informative abstracts revisited. **Ann Intern Med** 113:69–76, 1990.